Mesenchymal Characterization: Alternative to Simple CTC Detection in Two Clinical Trials

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Abstract. *Background: Breast cancer is one of the most common malignancies in women. Approximately 25% of patients with early-stage disease will develop metastatic recurrence. Two clinical trials were undertaken in order to detect circulating tumor cells (CTCs) in primary breast cancer. Patients and Methods: Four-hundred patients with early breast cancer were enrolled in the trial. After enrichment from their peripheral blood, their CTCs were characterized by gene expression of cancer cell markers. Results: CTCs had a predominant epithelial phenotype in 8.75% of patients and de-differentiated characteristics (mesenchymal, stem phenotypes alone or both) in 37.6%. Conclusion: Tumor epithelial cells undergoing epithelial–mesenchymal transition give rise to cells with mesenchymal aggressive phenotype. Detection of mesenchymal and cancer stem cells, which are tumor-initiating cells, is more relevant than simple counting of CTCs to assess their presence in the blood of patients with breast cancer. This study will be the basis for future evaluation of the outcome of the disease and the prognostic value of early-detected CTCs.*

Detection of circulating tumor cells (CTCs) has been recently developed and can be considered as a prognostic tool (1). A limited number of markers are currently used for the isolation (cell surface antigens) or detection (antigens or mRNA) of CTCs. One such marker is the epithelial cell adhesion molecule (EpCAM) (2); others are more cancer-specific, such as human epidural growth factor receptor 2 (HER2) and mucin 1 (MUC1) for breast carcinoma. CTCs are generally counted by using the CellSearch system (Veridex, Warren, New Jersey, USA). In this technique, blood sample is enriched for CTCs by EpCAM-coated immunomagnetic beads. CTCs are then stained for cytokeratins (CK8, CK18 and CK19), for CD45 to eliminate white cells, and with 4',6-Diamidino-2-Phenylindole (DAPI) nuclear counterstain for essessment of their viability. Finally, they are enumerated by at least two pathologists. This method was cleared by the US Food and Drug Administration (FDA) but not endorsed by the American Society of Clinical Oncology (ASCO). Due to the use of a sole antibody, CTCs can escape the enrichment and detection phases, as EpCAM expression is decreased in mesenchymal cells (3). In one-third of patients, CTCs are undetected by conventional methods (4). This impairment prompted the development of methods where CTCs are qualified by molecular biology techniques, revealing expression of specific genes (5). AdnaGen technology is one of those techniques (6). It is based on a combined mixture of antibodies (EpCAM and two epitopes of MUC1) to trap CTCs which are defined by expression of genes involved in epithelial–mesenchymal transition (EMT). Most CTC detection trials were dedicated to metastatic breast cancer (7). CTCs were detected, depending on the stage of the disease and the undertaken methodology, in 8% to 95% breast cancer patients (8-12). Due to the range of results, conclusive relevance is still pending. However, these studies have shown the prognostic potential of CTCs in primary and metastatic breast cancer (13). Our preliminary results on primary breast cancer and CTCs arising from EMT were presented in Prague (June 13-17, 2011) at the TATAA Biocenter qPCR symposium (14). These results were confirmed by Kasimir-Bauer *et al.* (15). Today, the term CTCs encompasses all types of cells which are considered as foreign entities in the blood, exhibiting cancerous characteristics. This term does not describe the diversity of CTC subpopulations. Among them, cancer stem and mesenchymal cells have to be taken into account. Their features are invasiveness linked to increased cell motility and their capability to avoid apoptosis, anoikis, and general immune defense (16-19). Upon hypoxia, epithelial cancer cells can modify their phenotype. This phenomenon depends on pleitropic cytokines such as

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transforming growth factor β (TGF-β) (20). The latter is induced by hypoxia-inducible factor 1α (HIF1α) (21). The signaling pathway induces loss of the epithelial polarity of cells, which undergo cytoskeletal remodeling. Expression of some proteins which are involved in adhesion structures are inhibited. Numerous experiments on this transition have demonstrated a decrease of E-cadherin and an increase of N-cadherin and vimentin (22-24). Thus, cells acquire a migratory phenotype and exhibit mesenchymal features. Epithelial cells and mesenchymal cells can exist simultaneously, both types being CTCs. Hence, a clinical trial was developed to establish the percentage of patients carrying these cells at the time of early diagnosis of breast cancer and before and after surgical eradication of the primary tumor. In this protocol, 400 patients were included and CTCs were evidenced by three markers: HER2, MUC1, epithelial glycoprotein 40 (GA733-2). Moreover, we analyzed 130 clinical samples belonging to this cohort to detect mesenchymal and stem cells by using more specific EMT markers: transcription factor TWIST1, protein kinase B (AKT2), phosphatidylinositol 3-kinase A (PI3KA) and aldehyde deshydrogenase 1 (ALDH1).

Materials and Methods

Patients. Four-hundred patients (T1/T3, N^-/N^+, M0) were enrolled between 2009-2011. Patients were selected provided they fulfilled the following criteria: age 40-75 years; breast cancer diagnosis confirmed by a pathologist's analysis of the primary tumor; absence of bone, visceral, cerebral metastasis (controlateral breast mammography, liver ultrasonography and entire body-bone scanning). Axillary lymph node invasion was assessed. These patients were treated by tumorectomy or mammectomy; axillary clearance was added when required. Characteristics of the primary tumor at the time of diagnosis are shown in Table I. All blood samples were obtained after informed consent. The protocol was conducted at the Department of Gynecological Surgery (Private Hospital Clinique le Colombier) and Astralab Laboratory (Department of Specialized Clinical Analyses) in Limoges France. The study was performed with approval of an appropriate Local Ethics Committee (Comité de protection des personnes Sud-Ouest et Outre-mer IV. France) and was in compliance with the Helsinki Declaration. This cohort enabled us to realize two trials: the totality of patients (400) was incorporated in a first protocol and the final 130 patients were also included in a second protocol.

Sampling of biological material. Blood samples of 7 ml were collected for cellular enrichment with AdnaCollect tubes (AdnaGen AG, Langenhagen, Germany) before surgery and at least 3 weeks after surgery. Blood collections were performed before any drug therapy. Two tubes of 7 ml were necessary when patients were enrolled into the second trial. Samples were stored, shipped in the dark at 4-8˚C and were analyzed within 24 h. Blood collection tubes contain EDTA and a chemical agent to prevent illegitimate RNA expression.

CTC selection and detection. Selection and detection of CTCs were performed as described elsewhere (5). The following AdnaGen kits were used: AdnaTest Breast Select and Detect, AdnaTest EMT-1/Stem Cell Select and Detect, according to the suppliers instructions (AdnaGen AG, Langenhagen, Germany). Steps are briefly described. For semi-quantitative reverse transcription and polymerase chain reaction (RT-PCR), Sensiscript and HotStarTaq from Qiagen GmbH (Hilden, Germany) were used and the housekeeping gene was β actin. Thermal profiles are those recommended by the supplier. Visualization of PCR fragments was carried out with a bioanalyser (2100 Bioanalyser; DNA 1000 LabChip; Agilent Technologies; Santa Clara; CA). Peaks were considered to be positive when concentrations were ≥0.15 ng/μl (according to AdnaGen indications).

Protocol 1: Tumor cell enrichment was realized by using *AdnaTest Breast Select*. The immunomagnetic beads were coated with three antibodies, one to EpCAM and two others to MUC1. Then, tumor-associated mRNA expressions were analyzed by *AdnaTest Breast Detect* kit. The following transcripts were separated by capillary electrophoresis: *HER2*, *MUC1* and *GA733-2* with base pairs of size 270, 293 and 395 bp, respectively.

Protocol 2: AdnaTest EMT-1/Stem Cell Select avoided an excess contaminating leukocytes by a special washing buffer procedure. Expressions of tumor-associated mRNA were depicted by *AdnaTest EMT-1/Stem Cell Detect* kit. The following transcripts: *TWIST1, AKT2, PI3KA* and *ALDH1* (203 bp, 306 bp, 595 bp, 165 bp, respectively) were revealed by capillary electrophoresis. To the supplied method, we added *CD44* and the polycomb group protein BMI1 (*BMI1*) as subsidiary markers of stemness. For each one, the RT sample, previously obtained, was amplified by singleplex PCR. Sequences of primers were *CD44* forward: GCCCAATGCCT TTGATGGACC and reverse: GCAGGGATTCTGTCTGTGCTG; and *BMI1* forward: CATTGTCTTTTCCGCCCGC and reverse: CAAAG CACACACATCAGGTGGG. The thermal profile used for *CD44* PCR was as follows: after 15 min denaturation at 95˚C, 33 cycles of PCR were carried out by denaturation at 94˚C for 30 s, annealing/extension at 59˚C for 30 s, and elongation for 72˚C for 30 s. Termination of the PCR reaction was subsequently carried out at 72˚C for 5 min followed by storage of the sample at 10˚C. The procedure for *BMI1* was as follows: after 15 min denaturation at 95˚C, 36 cycles of PCR were carried out by denaturation at 94˚C for 30 s, annealing/extension at 59.7˚C for 30 s, and elongation for 72˚C for 30 s. Termination of the PCR reaction was subsequently carried out at 72˚C for 5 min followed by storage of the sample at 10˚C. The primers generate fragments of the following sizes: (*CD44*, 257 bp and *BMI1*, 132 bp). *CD44* and *BMI1* expressions were considered positive when the transcript concentration was above 0.50 ng/μl. Blood collected from 20 healthy donors was investigated to determine this cut-off value.

Statistical analysis. Statistical analyses were performed using XLStat2011 software (Addinsoft; Paris; France). The Chi-square test was used to establish a relationship between CTC detection and tumor characteristics and lymphatic invasion.

Results

Protocol 1: Patients' characteristics. A total of 400 patients (aged from 40 to 75 years, average 66 years of age) were included in the trial. They were studied at the time of newly-diagnosed breast cancer and patients were enrolled after elimination of bone visceral and cerebral metastasis, whether

Table I. *Tumor characteristics for 400 patients included in protocol 1. CTC-positivity is indicated for each category of breast cancer and before or after surgery.*

Patient cohort characteristics		Cohort	CTC positivity	Only before surgery	Only after surgery	Both before and after surgery
Number		400	35	15	12	8
Histology	Invasive ductal carcinoma	314	28	10	10	8
	Invasive lobular carcinoma	47	3	1	2	0
	Other	39	4	4	0	0
Molecular characteristics	Luminal A	309	25	8	10	7
	Luminal B	29	3	0	2	1
	Triple negative	33	2	2	0	0
	HER2 overexpression	16	1	1	0	0
	ND	13	4	4	0	0
Tumor size	T1	180	14	5	6	3
	T2	171	16	7	5	4
	T3 and T4	38	4	2	1	1
	ND	11	1	1	0	0
Lymph node status	N^-	268	27	11	10	7
	N^+	116	7	3	2	1
	ND	16	1	1	0	0
Grading	I	60	10	3	4	3
	II	235	16	6	6	4
	III	92	8	5	2	1
	ND	13	1	1	0	0
Receptor status estrogen	ER^-	54	3	3	0	0
	ER^+	339	32	12	12	8
	ND	7	0	0	0	0
Receptor status progesterone	PR^-	100	7	4	1	2
	PR^+	293	28	11	11	6
	ND	7	0	0	0	0

ND: Not determined.

they had axillary lymphatic node invasion or not. Among the cohort, 87% of patients had T1 or T2 tumors. Most of the patients had no positive axillary lymph nodes (67%). The most common molecular characteristic phenotype, assessed on estrogen receptors (ER), progesterone receptors (PR) and HER2 expression of the primary tumor, was hormone receptor positivity. Luminal A type breast cancer was observed in 77%, luminal B in 7%, triple negative (TN) in 8% and HER2 was overexpressed in 4% of breast cancer cases (Table I).

Incidence of CTCs. In 35 patients, CTCs were detected at least once (either before and/or after surgery). Thus 8.75% of the total cohort expressed at least one marker. Out of the 400 patients, 359 were submitted to the two series of analyses before and after surgery. The analysis of markers for the 35 CTC-positive patients gave the following positivity rates: GA733-2, 42%; MUC1, 65%; HER2, 26%. In eight patients, CTCs were detected both pre- and post-operatively. Out of the 23 patients found to be CTC-positive before surgery, eight remained positive. Thus, 23% of the initially CTC-positive patients failed to clear cancer cells from their blood despite suppression of the primary tumor. Out of the 20 patients found to be CTC-positive after surgery, 12 were cases of new detection. These data are presented in Table I. There was a discrepancy between the HER2 phenotype of CTCs and that of the primary tumor. Effectively, out of four patients with HER2-positive primary tumor, only two had CTCs bearing the HER2 receptor. In seven cases of HER2-negative tumor, the corresponding CTCs were positive for HER2. This result showed a high incidence of conversion for the EGF receptor. CTC positivity was distributed as follows: 25 luminal A, 3 luminal B, 1 HER2-overexpressing and 2 TN. Four breast tumors were not molecularly characterized. CTCs are often detected in luminal phenotypes. However, the imbalance between the different types of tumors in the studied cohort is not sufficient to support this assertion. Statistical studies indicated that CTC detection is independent of the primary tumor characteristics. This non-correlation between CTCs and parameters of the tumor was demonstrated by *p*-values, which in all cases were not less than 0.05.

Table II. *Tumor characteristics for 130 patients included in protocol 2. Positivity of mesenchymal or/and stem cells is indicated for each category of breast cancer before surgery.*

Patient cohort characteristics		EMT cohort	ddCTC positivity	EMT+ alone	Stem+ alone	Both EMT+ Stem+
Number		130	49	18	17	14
Histology	Invasive ductal carcinoma	100	39	13	17	9
	Invasive lobular carcinoma	16	4	2	0	2
	Other	14	6	3	0	3
Molecular characteristics	Luminal A	105	40	13	14	13
	Luminal B	6	1	1	0	0
	Triple negative	9	5	2	3	0
	HER2 overexpression	8	1	1	0	0
	ND	2	2	1	0	1
Tumor size	T1	62	24	11	7	6
	T2	50	17	3	8	6
	T3 and T4	12	5	2	2	1
	ND	6	3	2	0	1
Lymph node status	N^-	84	32	12	9	11
	N^+	37	11	3	7	1
	ND	9	6	3	1	2
Grading	I	25	11	4	3	4
	II	71	25	9	8	8
	III	24	8	3	5	0
	ND	10	5	2	1	2
Receptor status estrogen	ER^-	18	7	2	5	0
	ER^+	108	40	15	12	13
	ND	4	2	1	0	1
Receptor status progesterone	PR^-	35	11	2	5	4
	PR^+	91	36	15	12	9
	ND	4	2	1	0	1

EMT: Epithelial–mesenchymal transition; ddCTC: de-differentiated circulating tumor cell; ND: not determined.

Protocol 2: Blood samples from 130 patients, submitted to the CTC analyses described above, were tested for CTC presence by using EMT and stemness markers. All patients were studied at the time of newly-diagnosed breast cancer. Table II describes clinical and pathological characteristics of the tumors. We distinguish these cells by calling them de-differentiated CTCs (ddCTCs), as markers used for their detection are implicated in their mesenchymal or stemness status.

EMT-stemness detection. Among 130 patients, 49 had ddCTCs expressing at least one EMT marker or *ALDH1* mRNA. Thus 37.6% of samples were positive for ddCTCs (Table II). Moreover among this population, 36.7%, 34.6% and 28.6% exhibited EMT cell, stem cell or both EMT and stem cell markers respectively. The analysis of each marker for the 49 ddCTC-positive patients gave the following positivity rates: *TWIST1*, 8.2%; *PI3KA*, 61.2%; *AKT2*, 10.2%; and *ALDH1*, 63.2%. When analyses detected at least one EMT marker or *ALDH1*, the sample was further examined for *BMI1* and *CD44*. For these two markers, we applied a transcript cut-off value of 0.50 ng/μl (specificity of the cut-off is more than 90%, as confirmed in 20 healthy donor samples). Thus, ddCTCs detected by mesenchymal markers and/or *ALDHI* were 63% and 40% positive for *BMI1* and *CD44*, respectively. Among 84 N^- patients, 32 had ddCTCs, and of 37 N^+ patients, only 11 had ddCTCs. Statistical studies indicated that ddCTC detection is an independent factor of the other pronostic factors based on the pathologist's analysis. No *p*-value was less than 0.05. In a previous report, we noticed a correlation between the presence of ddCTCs and lymph node positivity with $p<0.05$ (5). This value is at the limit of significance, indicating a trend, and this correlation disappeared as the number of patients increased (from 61 to 130).

Discussion

In the first protocol, detection of CTCs led to discovery of a small rate of CTC positivity at the time of early-breast cancer diagnosis, when cancer cells were detected on the basis of GA733-2, HER2, MUC1 marker expression

(8.75%). These results seem low when compared to those previously published, which could be due to the use of different technologies. However, Molloy *et al*. and Banys *et al*. who conducted trials comparable to ours, described CTC positivity with 7% and 12% rates, respectively for their patients (8, 9). To explain such a low percentage of patients with CTCs, one must bear in mind that CTC isolation is based on epithelial characterized antibodies of cells (25). Moreover, at the detection step, the markers are limited to some of the total CTC populations. It can be expected that detected cells are epithelial cancer cells and that those loosing the epithelial marker (EpCAM) are missed. Cancer cells in the blood arise from the primary tumor and two types of processes could be implicated: passive or active delamination. In the first phenomenon, escape of cancer cells occurs as clusters and cells retain their epithelial characteristics. Collective epithelial cell migration is an integral part of development and cancer progression. Apicobasal polarity is maintained when cell strands or sheets leave the primary tumor (26, 27). The lifespan of such cells is limited by anoikis and solely their presence in the blood is an indicator of the existence of a primary tumor. Their evidence does not necessarily indicate that they will survive and grow but they can testify to the existence of hidden cell subpopulations not detected by the method, coming from an active delamination process during EMT (28). The data reported here also demonstrated that out of the 23 CTC-positive patients, eight had persistence of cancer cells in the blood even three weeks after removal of the primary tumor. Moreover, regarding CTC-negative patients before surgery, 12 became CTC-positive four weeks after removal of the primary tumor. This positivity, demonstrating that cancer cells are not always cleared by surgery, can be explained by the presence of a secondary cancer site which releases CTCs into the blood. Many questions still remain about this positive recurrence. The major concern is whether micrometastases exist a long time before they are revealed by their growth. 12-37% of small breast tumors (<1 cm), have already metastasized at diagnosis (29, 30) and some data suggest that systemic dissemination of tumor cells occurs at early stages of tumor development (31). Based on these considerations, we started the second protocol to detect cells arising from EMT and which are not evidenced by the previous method.

An oncological type of EMT is able to convert differentiated epithelial cancer cells into migratory mesenchymal cancer cells. EMT is associated with poor clinical outcome in breast cancer (32, 33). PI3K signaling plays a key role in inducing and maintaining EMT. The most important downstream effector of PI3KA is AKT. Moreover, migratory mesenchymal cells acquire some stem cell characteristics and can be detected by using *ALDH1*, *BMI1* and *CD44*. Numerous transcription factors have been implicated in regulating the EMT status, such as TWIST1. Thus these markers are relevant for the detection of mesenchymal cells. By using these, we detected 49 patients out of 130 who had ddCTCs in the blood at the time of early diagnosis of primary breast cancer. These ddCTCs can be distinguished into three phenotypes: mesenchymal, stem, or mixed status (EMT and stemness). Even patients with node-negative invasion had ddCTCs: 32 out of 84 cases. This positivity is independant of tumor clinicopathological features. It would be of interest to test blood of these patients after surgery and during progression-free survival to determine whether these CTCs are implicated in the recurrence or metastases occurring in 25% of women at 5 years. The stemness characteristics of these cells, particularly dormancy, would be at the origin of the "waking-up" of the disease. We cannot emphasize enough that the selected method is crucial in order to truly detect cancer cells in the blood. Most of the conventional methods are not able to delineate the subpopulations issued from EMT. In the cohort of 400 patients, we only found positivity for 8.75% of samples, whereas 37.6% were positive when we examined EMT characteristics. Thus it seems evident that the second method is more appropriate to analyse CTCs in breast cancer at the time of early diagnosis. Thus an EMT signature of CTCs may help to stratify early-stage breast cancer. However, additional studies are required to determine the prognostic potential of EMT associated with CTCs. Consequently, a follow-up of at least five years may be requiered to include mature outcome data.

Conclusion

The aim of the study was to assess CTCs expressing mesenchymal markers in patients with primary breast cancer. Due to frequent loss of epithelial antigens by CTCs, the most invasive cell populations are hidden. Detection has to be improved to identify CTCs which have undergone EMT. The two protocols demonstrated that initiating tumor cells which are subpopulations of ddCTCs are often missed. Such a result is of importance because it is these cells which support invasion and metastases, and potentially support the genesis of residual disease. It has been demonstrated that ddCTCs are resistant to chemotherapy and radiotherapy. Neoadjuvent therapy to reduce the volume of tumor before surgery could concentrate these cells in the primary tumor. Thus it might be necessary to establish a follow-up of ddCTCs to monitor how they are cleared from the blood. Methodologies to identify ddCTCs should allow for an accurate screening of new molecules which would be able to target or reverse EMT.

Competing Interests

The Authors declare that they have no competing interests.

Authors' Contributions

All contributors participated equally to this study. All authors read and approved the final manuscript.

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